The invention also relates to antibodies, preferably monoclonal antibodies, which are directed to the polypeptides according to this invention.

Finally, it is an object of the invention to provide a pharmaceutical preparation comprising a polypeptides defined above and below, where appropriate, together with suitable excipients, carriers and other active ingredients.

Detailed Description

(A) ISOLATION AND PURIFICATION

The histidine protein phosphatase was isolated from rabbit liver.

The liver preparation starts from about 110 g of material cut into small pieces. It is mixed with homogenization buffer (220 ml of 30 mM triethanolamine/hydrochloric acid pH 7.5, 1 mM ethylenediaminetetraacetic acid, 300 mM sucrose, 0.1 mM benzamidine, 0.1% 2-mercaptoethanol) and comminuted in a homogenizer while cooling in ice. After a first centrifugation (10 min at 3800 g, 4°C), the supernatant is recentrifuged (1 h at 48,000 g, 4°C). This supernatant is filtered through gauze and frozen in aliquots of about 20 ml at -80°C.

Column chromatographic separation methods

The histidine phosphatase was isolated by three purification steps (Figure 1).

1. Anion exchange chromatography

The crude liver extract is centrifuged once again (30 min at 48,000 g). The supernatant is loaded onto a Source Q30 (Pharmacia, Freiburg) column equilibrated with buffer A (20 mM triethanolamine/hydrochloric acid pH 8.0, 1 mM ethylenediaminetetraacetic acid, 0.1% 2-mercaptoethanol, 0.02% sodium nitrite). Elution takes place with 200 mM sodium chloride in buffer A at a flow rate of 1 ml/min.

2. Gel filtration

The active fraction (see activity determination) is stirred and cooled while solid ammonium sulfate is added (11.2 g to 17 ml). The pellet obtained by

centrifugation (20 min at 48,000 g, 4°C) is resuspended in buffer A and loaded onto a Superdex 75 26/60 1.6×60 cm (Pharmacia, Freiburg) column. The gel filtration takes place with the addition of 50 mM sodium chloride to buffer A at 1 ml/min.

3. Affinity chromatography

The active fraction from the gel filtration is diluted in the ratio 1:3 with buffer B (20 mM triethanolamine/hydrochloric acid pH 8.0, 0.1 mM ethylenediaminetetraacetic acid, 0.1% 2-mercaptoethanol, 0.02% sodium nitrite) and adjusted to 10 mM in magnesium chloride. The sample is loaded onto a Blue Sepharose 6 25×510 mm (Pharmacia, Freiburg) column. Elution takes place using buffer B containing 200 mM sodium chloride at a flow rate of 1 ml/min.

(B) SPECIFIC ACTIVITY DETECTION

The activity of the soluble histidine phosphatase is determined from the dephosphorylation of ^{32}P-labelled histidine-phosphorylated protein (CheA) as substrate. CheA is a recombinant bacterial histidine autokinase (Bilwes AM, Alex LA, Crane BR, Simon MI (1999) Cell 96:131-141); the C-terminal kinase domain phosphorylates the N-terminal His48. Free phosphate is produced in the reaction and is identified by thin-layer chromatography (polyethyleneimine cellulose plates, 0.5 M lithium chloride as mobile phase). Detection on the one hand via ammonium molybdate, and on the other hand by autoradiography (Figure 2). There was no phosphate transfer to other proteins, nor was there any cleavage of the substrate into peptide fragments. The product is phosphate, i.e. a phosphohistidine protein phosphatase is involved.

The purified protein shows time-, temperature-, pH- and protein-dependent histidine dephosphorylation of the ^{32}P-phosphorylated CheA (Figure 3).

Stability

The purified protein shows storage stability in the crude homogenate and in the partially purified fractions.

Enzyme assay

Substrate preparation: (^{32}P-labelling of CheA)

Recombinant CheA (5 µl) is mixed with 0.5 µl of 100 mM phenylmethylsulfonyl fluoride in dimethyl sulfoxide and 5 µl of 500 mM HEPES pH 8.0, 1 mM magnesium chloride. Addition of 108 µCurie of ^{32}P-g-adenosine triphosphate, 5 µl of 10 µM adenosine triphosphate and 50 µl of water is followed by incubation at 37°C for 3 h.

Activity determination

The substrate (10 µl of ^{32}P-CheA) is mixed with 10 µl of assay buffer (100 mM triethanolamine/hydrochloric acid pH 8.0, 0.1% 2-mercaptoethanol, 0.02% sodium nitrite) and the enzyme solution. The reaction takes place at 37°C in 30 min. Then 2 µl of 500 mM ethylenediaminetetraacetic acid and 126 µl of 1:1 methanol/acetone are added. After a centrifugation (5 min at 15,800 g), the supernatant is removed and measured in a scintillation counter.

The described phosphatase is thus differentiated from other protein phosphatases on the basis of

1) the specificity for phosphohistidine (Table 1), the histidine phosphatase did not hydrolyze e.g. p-nitrophenyl phosphate;
2) the activity not being inhibited by okadaic acid or vanadate (Table 2);
3) the molecular weight being considerably smaller.

Table 1. Hydrolysis by the novel histidin protein phosphatase

Azocoll	-
[γ-^{32}P] ATP	-
p-nitrophenyl phosphate	-
[γ-^{32}P] Ser/Thr-Casein	-
Tyr-xxx	-
[γ-^{32}P] His-nucleoside diphosphate kinase	-
[γ-^{32}P] His-cheA	+